Use of Biological Photoreceptors as Directly Light-Controlled Ion Channels

The present invention concerns the use of biological photoreceptors as directly light-controlled ion channels.

It has been known for some time that a large number of central processes in plant and animal cells are wholly or partially controlled via changes in the intracellular concentrations of certain ions, for example the proton concentration, and changes in the membrane potential and the ion gradients across the membrane. Accordingly, the elucidation of mechanisms of intracellular ion regulation, in particular pH regulation, and the mechanisms of voltage-dependent ion channels and ion transporters are the subject of extensive research activities. For such research, a rapid and simple measurement and/or manipulation of intracellular ion concentrations, in particular proton concentrations, and of electrical gradients and ion gradients, in particular proton gradients, across cell membranes is of great interest.

A basically very advantageous approach to making such measurements and/or manipulations possible would be the introduction of a light-controlled ion channel into such a membrane, in order to alter deliberately the membrane potential and/or the flow of certain ions, e.g. protons, through the membrane.

In the state of the art, a number of light-controlled ion transport systems are known. At the same time, a distinction is made between passive and active ion transport systems. In the passive ion transport systems, the actual transport molecules, the so-called ion channels, are in interaction with separate photoreceptor complexes and are controlled indirectly via these photoreceptors. Examples of such photoreceptor complexes interacting with an ion channel are the G-protein activating rhodopsins; see for example Müller, F., Kaupp, U. B., Signaltransduktion in Sehzellen, Naturwissenschaften 85 (1998) 49-61. Since these known passive ion transport systems require the combined action of several proteins and other components, there are problems in introducing them into other cells with the retention of their function, e.g. by co-expression of the relevant proteins in recombinant systems.

Examples of light-controlled active ion transport molecules in a cell membrane are the rhodopsins from archaea (archaebacteria). A known example of an archaeal rhodopsin is bacteriorhodopsin, consisting of a protein component, bacterioopsin, and a retinal linked via a Schiff's base. After excitation by light of suitable wavelength, bacteriorhodopsin transports protons through the membrane from the cell interior to the outside. As a rule, the ion transport proceeds slowly (< 100 H^+/ second) and the transport direction of the bacteriorhodopsin cannot be freely specified. *In vivo*, bacteriorhodopsin transports protons even against an existing electrochemical gradient, which is composed of a pH gradient and an electrical gradient $\Delta\Psi$. Such a light-controlled proton pump is a comparatively simple system. Bacteriorhodopsin and related retinal-binding proteins such as the chloride pump halorhodopsin have already been expressed heterologously and were capable of the light-driven active membrane transport of ions, e.g. H^+ or Cl^-, in the heterogeneous host.

However, for many purposes the known systems are unsuitable on account of their complexity or the predetermined transport direction.

One purpose of the present invention is to provide ways and means that allow rapid and simple measurement and/or manipulation of intracellular ion concentrations, in particular proton concentrations, and of electrical gradients across a cell membrane.

A further purpose is to provide a system which is suitable for the high throughput screening of biological molecules, in particular of pH-regulating membrane proteins and voltage-dependent membrane proteins.

The present invention solves these problems through the use of a new class of photoreceptors of previously unknown function, which are directly light-controlled passive ion transport systems (a light-controlled ion channel). This photoreceptor comprises an apoprotein which mediates ion conductivity, and a light-sensitive polyene covalently bound to the apoprotein, which interacts with the apoprotein and functions as a light-sensitive gate. Surprisingly, it has been possible according to the

invention to combine the advantages of a relatively simple directly light-controlled system with the advantages of a passive ion channel.

The apoprotein is a membrane protein with at least 5 transmembrane helices, which is capable of binding a light-sensitive polyene. Transmembrane proteins with 6 or 7 transmembrane helices are preferable. Transmembrane proteins with more than 7 helices, for example 8, 9 or 10 transmembrane helices, are however also covered by the invention. Furthermore, the invention covers transmembrane proteins which in addition to the transmembrane part include C- and/or N-terminal sequences, where the C-terminal sequences can extend into the inside of the lumen enclosed by the membrane, for example the cytoplasm of a cell or the inside of a liposome, or can also be arranged on the membrane outer surface. The same applies for the optionally present N-terminal sequences, which can likewise be arranged both within the lumen and also on the outer surface of the membrane. The length of the C- and/or N-terminal sequences is in principle subject to no restriction; however, apoproteins with C-terminal sequences not embedded in the membrane, with 1 to 1000 amino acids, preferably 1 to 500, especially preferably 5 to 50 amino acids, are preferred. Independently of the length of the C-terminal sequences, the N-terminal located sequences not embedded in the membrane preferably comprise 1 to 500 amino acids, especially preferably 5 to 50 amino acids.

The concept of the transmembrane helix is well known to the skilled person. These are generally α-helical protein structures, which as a rule comprise 20 to 25 amino acids. However, depending on the nature of the membrane, which can be a natural membrane, for example a cell or plasma membrane, or also a synthetic membrane, the transmembrane segments can also be shorter or longer. For example, transmembrane segments in artificial membranes can comprise up to 30 amino acids, but on the other hand also only a few amino acids, for example 12 to 16.

The ion channel according to the invention can in principle serve for the passive transport of all physiologically important ions. The best known ion transport systems transport Na^+, K^+, Ca^{2+}, H^+ or Cl^-. In a preferred embodiment, the ion channel according to the invention is a proton channel.

In an especially preferred embodiment, the proton channel includes an opsin protein or a derivative or fragment of a naturally occurring opsin protein as the apoprotein portion. Here, opsins refers to apoproteins which are covalently bound to a retinoid chromophore and display ion conductivity as soon as they absorb light. A molecule which contains a retinoid chromophore covalently bound to an opsin is referred to as rhodopsin.

A derivative of an opsin molecule which occurs naturally and functions as a light-switched ion channel is altered compared to the original by an exchange of one or several amino acids, by an insertion and/or deletion of one or several amino acids at one or several positions. Both the naturally occurring opsin molecules and also their derivatives should display an identity of at least 15 % with the sequence of the bacteriorhodopsin in the region of the 5 transmembrane helices which correspond to the helices 3 to 7 in the bacteriorhodopsin. An identity of 20 % or more between the derivative of a channel opsin and bacteriorhodopsin, based only on the region of the helices 3 to 7 in bacteriorhodopsin, is however preferred. On the other hand, the identity in the regions of the opsin derivative which do not correspond to the helices 3 to 7 can be far lower.

The term "identity" here refers to the degree of relatedness between two or more protein sequences, which is determined by the match between these sequences. The percentage identity is obtained as the percentage of identical amino acids in two or more sequences taking account of gaps and other sequence features.

The identity of mutually related protein molecules can be determined by means of known procedures. As a rule, special computer programmes with algorithms taking account of the particular requirements are used. Preferred procedures for the determination of the identity first generate the greatest match between the sequences under investigation. Computer programmes for the determination of the identity between two sequences include, but are not restricted to, the GCG programme package, including GAP (Devereux et al., 1984); Genetics Computer Group University of Wisconsin, Madison, (WI)); BLASTP, BLASTN and FASTA

(Altschul et al., NCB NLM NIH Bethesda MD 20894; Altschul et al., 1990). The well-known Smith Waterman algorithm can also be used for the determination of identity.

Preferred parameters for the sequence comparison include the following:

Algorithm: Altschul et al., 1990 (Basic local alignment search tool, J. Mol. Biol. 215, 403-410) (=BLAST)
Comparison matrix: BLOSUM 62 (Henikoff and Henikoff, 1992, Amino acid substitutions from protein blocks. PNAS 89, 10915-10919)
Agreement (matches) = variable
Non-agreement (mismatch) = variable
Gap value: open 10
Gap length value (gap length penalty): 1

The GAP programme is also suitable for use with the above parameters. The above parameters are the standard parameters (default parameters) for protein comparisons.

Further examples of algorithms, gap opening values (gap opening penalties), gap extension values (gap extension penalties), and comparison matrices including those mentioned in the programme handbook, Wisconsin Package, Version 9, September 1997, can be used. The choice will depend on the comparison to be performed and also on whether the comparison is performed between sequence pairs, in which case GAP or Best Fit are preferred, or between a sequence and an extensive sequence database, in which case FASTA or BLAST are preferred.

For the active proton pump bacteriorhodopsin, it is known that $Asp(D)^{96}$ is an amino acid essential for the proton pump function. Further, the following 16 amino acids of the bacteriorhodopsin are involved in the proton network:

F^{42}, T^{46}, Y^{57}, R^{82}, D^{85}, T^{89}, L^{93}, T^{107}, W^{182}, Y^{185}, W^{189}, E^{194}, E^{204}, D^{212}, K^{216}, F^{219}

In the ion channel according to the invention, a different amino acid from "D" is at the position corresponding to the D^{96} of the bacteriorhodopsin sequence. E^{204} is preferably replaced by S. In one embodiment, however, at least 8 of the other 15 amino acids are retained identical or only altered by conservative exchange. The amino acids which should as far as possible be retained identical are preferably T^{46}, Y^{57}, R^{82}, T^{89}, T^{107}, W^{182}, E^{194}, D^{212} and K^{216}. Conservatively exchanged amino acids are preferably F^{42}, D^{85}, L^{93}, Y^{185}, W^{189} and F^{219} The skilled person knows here that for a conservative exchange an amino acid is selected which is functionally similar to the amino acid to be exchanged. Thus exchanges are normally effected within the following groups:

(a)	Ala (A)	Ser (S)	Thr (T)	Pro (P)	Gly (G);
(b)	Asn (N)	Asp (D)	Glu (E)	Gln (Q);	
(c)	His (H)	Arg (R)	Lys (K)		
(d)	Met (M)	Leu (L)	Ile (I)	Val (V)	and
(e)	Phe (F)	Tyr (Y)	Trp (W).		

Based on the amino acids stated above, preferred exchanges are the following: F42Y, D85E, Y185F, W189F and F219W.

In a further preferred embodiment, one or several more of the following positions, based on bacteriorhodopsin, are contained in the ion channel according to the invention: Y^{83}, W^{86}, P^{91}, G^{122}, P^{186} and G^{195}.

In a further preferred embodiment, a passive proton channel according to the invention contains an apoprotein with the consensus sequence L(I)DxxxKxxW(F,Y). Amino acids given in brackets can in each case replace the preceding amino acid. This consensus sequence is the motif surrounding the retinal-binding amino acid lysine. In bacteriorhodopsin, the "K" at position 6 of the consensus sequence corresponds to K^{216} in the 7^{th} helix of the bacteriorhodopsin.

In a preferred embodiment, the ion channel includes an apoprotein from lower eukaryotes. The group of the lower eukaryotes includes for example algae, protozoa, ciliates and yeasts.

Especially preferred here are motile green algae, in particular Chlorophyceae. Apoproteins from Volvocales are of particular interest here. In the most preferred embodiment, the apoprotein is an opsin protein from *Chlamydomonas reinhardtii.* Further green algae with preferred embodiments can be found among the Ulvophytes such as *Acetabularia* and *Ulva*. Further preferred embodiments are opsins from Prasinophyceae, for example *Pyramimonas* and *Platymonas (Tetraselmis*). Other preferred forms derive from the kingdom of the Dinophytes with the individual class of the Dinophyceae and for example the members *Gymnodinium splendens, Gyrodinium dorsum, Peridinium balticum* and *Gonyaulax*.

In a further preferred embodiment, the opsin functioning as a light-controlled ion channel is derived from a protozoon, a bacterium or an archaebacterium.

In a further preferred embodiment, the opsin functioning as a light-controlled ion channel is derived from fungi such as *Neurospora crassa, Fusarium sporotrichioides* and *Leptosphaeria maculans,* or *Chytridiomyceten* such as for example *Allomyces reticulatus,* or from ciliates such as *Fabrea salina* or *Paramecium bursaria* or from Foraminifera such as *Amphistegina radiata.*

According to the invention, two different proteins of known sequence were functionally expressed from *Chlamydomonas reinhardtii* and for the first time identified as passive ion transport systems. These are Channelopsin1 (= CHOP-1; also called Chlamyopsin-3 = COP3) and Channelopsin2 (= CHOP-2; also called Chlamyopsin-4 =COP4).

The CHOP-1 protein has a molecular weight of 76 kD and a length of 712 amino acids. It was identified on the basis of overlapping partial cDNA sequences in a *C. reinhardtii* EST database (Asamizu et al., *DNA Research* 7, 305-7 (2000)). Its amino acid sequence is shown in Fig. 1 of the present application. The core protein (amino

acids 76–309) includes 7 hypothetical transmembrane segments with 15-20% homology to the sensory archaeal rhodopsins, the ion transporters bacteriorhodopsin (BR) and halorhodopsin (HR), and to an only recently identified rhodopsin from the fungus *Neurospora crassa* (NOP1). Quantitatively, these homology levels are admittedly relatively low, however, on comparison with BR, those amino acids which define the retinal binding site and the H^+ transport network in BR are specifically conserved. The consensus motif LDxxxKxxW observed suggests that in CHOP-1 K^{296} is the retinal-binding amino acid. 9 out of 22 amino acids which are in direct contact with the retinal in bacteriorhodopsin are identically retained in CHOP-1 and 4 others reflect only conservative changes ((Fig.1); Nagel et al., in preparation).

Detailed studies of the light-controlled ion transport function of the CHOP-1 protein in oocytes from *Xenopus laevis* showed that the transported ions are protons (Fig. 3), and moreover that the ion transport is of a purely passive nature (Fig. 4a-c). The induced photocurrent and hence the ion transport is dependent on the wavelength of the excitant light and reaches a maximum at 500 nm (Fig. 4d).

Analogous experiments with two shorter fragments of the CHOP-1 protein, which included the amino acids 1-346 and 1-517 respectively, yielded results which were essentially identical with those for the full-length CHOP-1 protein. This demonstrates that a large part of the carboxy terminal region of the CHOP-1 protein is not necessary for the ion transport function.

The CHOP-1 protein from *C. reinhardtii* is the first identified example of a new directly light-controlled passive ion transport protein. Structurally and/or functionally similar rhodopsin proteins also occur in other microalgae and in gametes and zoospores of macroalgae and possibly also in other organisms.

The second protein identified as an apoprotein of a light-switched ion channel is Channelopsin2 (CHOP-2), whose sequence comprising 737 amino acids is also shown in Figure 1. It displays a homology of 52.7 % to CHOP-1. The amino acids important for transport identified via the homology between BR and CHOP-1 and model calculations are also largely conserved in CHOP-2. For this rhodopsin also, a

light-switched passive proton conductivity was for the first time demonstrated by expression in *Xenopus* oocytes. The ion channel formed with CHOP-2 as apoprotein differs from that formed with CHOP-1 in terms of its unit conductivity, its inactivation under prolonged illumination and the shape of the current-voltage curve. Channelrhodopsin-2 (ChR2), which is made up of the protein Channelopsin-2 (CHOP-2) and retinal, is a light-controlled cation channel, which is permeable for example to Li^+, Na^+, K^+, Ba^{2+}, Sr^+ and Ca^{2+}, but not to Mg^{2+}. The maximum for the excitant light lies at 440-480 nm.

Similarly to the case of the CHOP-1 protein, it could also be shown here that a shortened form of CHOP-2 which contains the amino acids 1-315 likewise forms a functional light-switched ion channel (Figs. 5-7).

CHOP-2 and or CHOP-2-315 can be used for the depolarisation of cells (see Fig. 7) or for Ca^{2+} uptake (Fig. 6) or for both.

In the context of the present invention, with knowledge of the nucleotide and amino acid sequence and the calculated 3D structure of the natural CHOP-1 and CHOP-2 protein the skilled person is capable of preparing derivatives of the original amino acid sequence by exchange and/or insertion and/or deletion of one or several amino acid(s), which have wholly or partially retained the ion transport properties of the original protein or differ significantly with regard to ion specificity, inactivation, alignment, etc. Derivatives here are defined as stated above.

Active ion transport systems can be clearly and unequivocally distinguished electrophysiologically from passive systems as described below. In the light, passive light-controlled ion channels, which are an object of the invention, increase the conductivity of membranes into which they are incorporated, compared to the dark state. The ion currents which arise on illumination (photocurrents) are carried by the ion species which are conducted by the light-controlled ion channel. With equal concentration of the ions conducted (for CHOP-1 protons; for CHOP-2 for example protons) on both sides of the membrane (symmetrical conditions), the direction of the photocurrent is determined solely by the applied membrane potential. In the case of a

positive charge carrier, with a negative potential (negative in the cell), an inward current takes place with movement of the positively charged ions into the cell, and with a positive potential (positive within) an outward current with movement of the positively charged ions out from the cell. With a symmetrical distribution of the transported ions, the current-voltage curves of a light-controlled ion channel according to the invention always pass through the origin (reversal potential E_H=0). However, the current-voltage curves of the light-controlled ion channels according to the invention are not necessarily linear. In the preferred embodiment of CHOP-1 for example, the current is conducted better in the inward direction than in the outward direction (inward rectifier, see Figs. 2 and 3).

In contrast to this, in all active transport systems (such as for example bacteriorhodopsin), with symmetrical ion distribution the current-voltage curve never passes through the origin. The establishment of an unequal distribution of the conducted ions between inside and outside results in a displacement of the current-voltage curves along the voltage scale in accordance with the Nernst equation, i.e. by 58 mV for monovalent ions and by 29 mV for divalent ions.

Hence by investigation of the current-voltage curves it is possible to establish for every light-controlled ion channel whether it is a passive or an active system.

The present invention thus also concerns derivatives and fragments of the CHOP proteins which have wholly or partially retained the ion transport properties of the original protein or even display them to an increased extent, and structurally and functionally similar opsins from other organisms, of natural origin or modified by the use of recombinant techniques, if they display the stated biophysical properties and like CHOP-1 und CHOP-2 can be used for the stated purposes.

Extensive ranges of experiments for the preparation and study of such derivatives led to the surprising result that the amino acid position 134 in the CHOP-2 protein is of particular importance for the function as light-switched ion channel. Thus for example, by exchange of histidine at position 134 for arginine a particularly active mutant of the complete CHOP-2 protein or of the shortened CHOP-2-315 protein can

be created. This exchange can be performed by standard procedures, for example by site-directed oligonucleotide mutagenesis. Further data suggest that Glu123 is involved in a light-induced deprotonation, which leads to a desensitisation of the ion channel. Thus the mutation of Glu123 to Asp (E123D) markedly increased the ratio of the transient photocurrent to the equilibrium photocurrent, while the mutation of Glu123 to Gln (E123Q) causes the peak of the transient photocurrent almost to disappear, but also leads to a marked diminution in the steady-state photocurrent.

The passive ion transport system according to the invention contains a light-sensitive polyene. This can for example be p-hydroxycinnamic acid, retinal or a retinal derivative. Preferred retinal derivatives are selected from the following group: 3,4-dehydroretinal, 13-ethylretinal, 9-dm-retinal, 3-hydroxyretinal, 4-hydroxyretinal, naphthylretinal; 3,7,11-trimethyl-dodeca-2,4,6,8,10-pentaenal; 3,7-Dimethyl-deca-2,4,6,8- tetraenal; 3,7-Dimethyl-octa-2,4,6-trienal; and 6-7, or 10-11 rotation-blocked retinals with 4-, 5-, 6- or 7-member ring bridges. Especially preferred, however, is a 10-12-five-member ring-bridged retinal (Takahashi et al. FEBS Lett. 314, 275-279).

After light absorption and isomerisation of the polyenes, a structural alteration in the protein (opsin) takes place in the light-controlled ion channels according to the invention, and hence the opening of the ion-conducting channel, which links the intracellular with the extracellular side of the membrane. This event differs fundamentally from the situation in the known ion pumps, in which the extracellular proton-conducting half-channel (EC) is never conductively connected to the intracellular half-channel (IC). In bacteriorhodopsin, the Schiff's base of retinal is conductively connected to the extracellular side in the early M intermediate of the reaction cycle (photocycles), and conversely in the late M state it is connected to the intracellular side.

The light-controlled ion channels according to the invention can be incorporated into a membrane, for example the plasma membrane of a cell, and used to alter the membrane potential rapidly and in a defined manner through illumination, which is very helpful for the elucidation of the mechanisms of voltage-dependent ion channels or ion transporters. Further, the possibility thus arises of altering the intracellular

levels of these ions rapidly and in a defined manner by targeted light-controlled ion transport.

In the case of the CHOP-1 protein, this means a targeted, non-invasive alteration of the intracellular pH value, which is of use for the elucidation of the mechanisms of intracellular pH regulation or the influence of transient pH changes on endogenous cell proteins.

With knowledge of the extracellular pH, the intracellular pH directly under the membrane or a pH gradient across the membrane can be rapidly and accurately measured through the measurement of the reversal potential of the light-induced CHOP-1 mediated proton conductivity. For this, the reversal potential is determined with the "Voltage Clamp" measurement used in Figs. 2-4, and the cells are thus calibrated. After this, the modulation of the membrane potential and/or the intracellular pH be effected non-invasively with light via the light-controlled ion channel according to the invention. The relevant measurements are performed rapidly and in a defined manner and are thus ideally suitable for modern HTS (High-Throughput-Screening) instruments, with which for example pH-regulated voltage-dependent membrane proteins such as ion channels can be tested in screening applications with high throughput. Abrupt changes in potential or pH can be induced with light in cell lines which contain ChR1 or related rhodopsins.

An especially interesting application is optoelectrical coupling by light-controlled modulation of the membrane potential. This application is based on a combination of the light-controlled passive ion channel with a light-controlled active ion transport system, e.g. an ion pump, wherein, a different wavelength is preferably used for the light-control of the passive ion channel than for the light-control of the active ion transport system. In a preferred embodiment, light of a wavelength of 650 nm is used for the activation of the proton pump bacteriorhodopsin and for building up a membrane potential and then light of a wavelength of 440 nm, which has an inhibitory action on bacteriorhodopsin, is used for the activation of the CHOP-1 proton transport system and for rapid dissipation of the potential.

Light-regulated ion channels can also be used in signal transfer from neuronal networks to microelectrode networks. In this way, transfer of electrical impulses from neurones to microcomputers is being attempted ("Interfacing of Nerve Cells and Semiconductors": Fromherz (2001), Physikalische Blätter 57, 43-48). Hitherto, the neurones had to be stimulated either via neurotransmitters or directly with micropipettes. Neurones which express ChR1, ChR2 or related light-controlled ion channels could be controlled with light.

A further application is the treatment of blind animals or in the final analysis people. There are a number of diseases in which the natural visual cells no longer function, but all nerve connections are capable of continuing to operate. Today, attempts are being made in various research centres to implant thin films with artificial ceramic photocells on the retina (E. Zrenner (2002) Science 295, 1022-1025.) These photocells are intended to depolarise the secondary, still intact cells of the retinal and thereby to trigger a nerve impulse (*bionic eyes)*. The deliberate expression of light-controlled rhodopsins such as ChR1 or ChR2 in these ganglion cells, amacrine cells or bipolar cells would be a very much more elegant solution and enable greater three-dimensional visual resolution.

The incorporation of the rhodopsin ion transport system according to the invention into the membrane of cells which do not express the corresponding opsin protein in nature can for example be simply effected in that, using known procedures of recombinant DNA technology, the DNA coding for this opsin is firstly incorporated into a suitable expression vector, e.g. a plasmid, a cosmid or a virus, the target cells are then transformed with this, and the protein is expressed in this host. Next, the cells are treated in a suitable manner, e.g. with retinal, in order to enable the linkage of a Schiff's base between protein and retinal.

In a preferred embodiment, this occurs in various yeasts such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe* or *Pichia pastoris* as already successfully performed for rhodopsins such as bacteriorhodopsin and/or bovine rhodopsin (Hildebrandt V. et al. (1989)), Genetic transfer of the pigment bacteriorhodopsin into the eukaryote *Schizosaccharomyces pombe,* FEBS Lett. 243(2):137-40; Najmoutin

G. et al., Heterologous expression of bovine opsins in *Pichia pastoris, Meth. Enzymol.* (2000) 315, 3-11).

The expression can also be effected in certain mammalian cell systems. This is effected either with episomal vectors as transient expression, preferably in COS cells (generated by infection of "African green monkey kidney CV1" cells) (Oprian et al. (1987) *Proc Natl. Acad. Sci* USA 84, 8874 ff.) or HEK cells ("*human embryonic kidney* cells", e.g. HEK293 cells, Reeves et al. (1996) *Proc. Natl. Acad. Sci USA 84*, 11487 ff.) or BHK-cells ("baby hamster kidney cells"), or in the form of stable expression (by integration into the genome) in CHO cells ("Chinese hamster ovary cells"), myeloma cells or MDCK cells ("Madine-Darby canine kidney cells") (Review in: Makrides SC (1999) Prot. Expr. Purif. 17, 183-202) or in Sf9 insect cells infected with baculoviruses (Jansen et al. (1988) *Mol Biol. Rep.* 13, 65 ff.).

To ensure or optimise expression, the coding DNA can also be suitably modified, for example by coupling with suitable regulatory sequences and/or by matching of the coding DNA sequence to the preferred codon usage of the chosen expression system.

Description of Diagrams:

Fig. 1

Fig. 1A: Amino acid sequence of Channelopsin1 (Chop1) SEQ ID NO: AF385748

Fig. 1B: Amino acid sequence of Channelopsin2 (Chop2) SEQ ID NO: AF461397

Fig. 1C: Amino acid sequence of bacterioopsin (Bop) from *Halobacterium salinarium* (BR). The *leader sequence*, which is cleaved off *in vivo* and for historical reasons is not counted in the numbering of the amino acids, is indicated in small letters. The amino acids essential for proton conduction are shown in bold letters.

Fig. 1D: Comparison of the amino acid sequences of CHOP-1 (SEQ ID NO: AF461397) and CHOP-2 (SEQ ID NO: AF461397) from *Chlamydomonas reinhardtii* with that of bacteriorhodopsin from *Halobacterium salinarum.* Amino acids, which are known to interact directly with retinal in BR (Lücke et al. (1999) Science 286, 255-260 and literature cited therein) are indicated by asterisks. Amino acid positions which are

the same in at least two sequences are backed in light grey. Amino acids which contribute to the H^+-conducting network in BR and the amino acids corresponding to these in the other opsins are white against a black background. For the His 173 of CHOP-1, it was shown in the context of the invention that it is involved in the proton conduction. # indicates the position of the retinal-binding lysine. The underlined amino acid indicate the 7 transmembrane helices of the core protein.

Fig. 1E: Amino acid sequence of the CHOP-2 core protein mutant CHOP2-315/-H134R), in which histidine at position 134 is replaced by arginine.

Fig. 2 Photocurrents which were recorded during the irradiation of oocytes with green or red light (500 $\pm$ 25 nm and 700 $\pm$ 25 nm respectively, 10^{22} photons, m^{-2} s^{-1}). holding potential (V_h) = -100 mV, light pulse indicated by the bar.

Bath solution = 96 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM MOPS, pH 7.5. A) An oocyte which was not injected with CHOP-1 RNA, irradiated with green light B) a CHOP-1 oocyte, irradiated with green light; C) the same oocyte as in B), irradiated with red light.

Fig. 3 Dependence of the light-induced inward current on the ion conditions and the potential.

a: Results with one (of five) characteristic CHOP-1 oocytes, shown in order of measurement (about 150 sec interval), V_h = -100 mV, green light as in Fig. 2. The solutions are buffered with 5 mM MOPS (pH 7.5) or MES (pH 6) or citrate (pH 5 and 4). Concentration in mM:

1. 100 NaCl, 2 $CaCl_2$, pH 7.5
2: 100 NaCl, 2 $CaCl_2$, pH 6.0 (reference)
3: 100 Na aspartate, 2 $CaCl_2$, pH 6.0
4: 100 NMG-Cl, 2 $CaCl_2$, pH 6.0
5: as in 2
6: 100 NaCl, 2 EGTA, 2 $MgCl_2$, pH 6.0
7: 200 sorbitol, 5 EGTA, pH 5.0
8: 200 sorbitol, 5 EGTA, pH 4.0

b: Current-voltage ratios of the photocurrents of Fig. 3a, see above for concentrations.

Fig. 4 Photocurrents which were recorded with variation of the external and internal pH value, and their dependence on the wavelength

a: Photocurrent at V_h = +40 mV. Results from one (of five) characteristic CHOP-1 oocytes in a bath solution of NaCl (100 mM, Track A: red light; Track B: green light; 10^{22} photons m^{-2} s^{-1}) or sodium butyrate (40 mM, +60 mM NaCl, Track C: green light) of pH 7.4

b: Current-voltage ratios of photocurrents in different bath solutions, which always contained 100 µM undissociated butyric acid:
■, 60 mM NaCl + 40 mM Na butyrate, pH 7.4; ▲, 84 mM NaCl + 16 mM Na butyrate, pH 7.0; ▼, 93.6 mM NaCl + 6.4 mM Na butyrate, pH 6.6; ♦, 97.4 mM NaCl + 2.6 mM Na butyrate, pH 6.2;

c: pH dependence of reversal potentials from b. The dotted lines show the theoretical relationship for a constant pH_i of 6.6 or 6.8 and -58 mV/pH. The dashed line shows the expected relationship for a gradient of -48 mV/pH difference and pH_i = 6.8 at pH_o = 7.4. The gradient of -48 mV/pH corresponds to a slowly decreasing pH_i (by 0.17 units per pH_o decrease of one unit). ■, pH dependence of reversal potentials from experiments with 5.4 mM of undissociated acetic acid (n =3). The dotted line shows the theoretical relationship for a constant pH_i of 5.5 and -58 mV/pH.

d: Wavelength dependence of the light-induced inward flow at pH_o 5.5 and - 40 mV. The photocurrents were standardised for an equal photon flow.

Fig. 5 Photocurrents which were recorded on illumination (wavelength 450 $\pm$ 25 nm) of CHOP2-315 expressing oocytes in Ringer's solution (110 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, 5 mM MOPS, pH 7.6) at +40 mV and -80 mV.

Fig. 6 Magnitude of the light-induced equilibrium inflow at -100 mV, pH 9, for 115 mM Li, Na, Cs, TMA, TEA or NMG and for 80 mM Mg^{2+}, Ca^{2+}, Sr^{2+} or Ba^{2+} (identical for both modifications CHOP2 and CHOP2-315)

Fig. 7 Depolarisation of an oocyte plasma membrane which expresses CHOP2-315, due to a light flash of 200 msecs

The following examples illustrate the present invention, without however limiting the invention to these

EXAMPLE I

Amplification of CHOP-1 DNA and Expression of
Functional CHOP-1 Photoreceptors in *Xenopus laevis*

A full-length CHOP-1 DNA, coding for the amino acids 1-712, and two shorter CHOP-1 DNAs, respectively coding for the amino acids 1-346 and 1-517 of CHOP-1, were amplified from a full-length cDNA matrix by PCR using a polymerase with correction function (pfu, Promega) and two start oligonucleotides (primers), which contained BamHI and HindIII restriction sites. The products were inserted into the vector pGEMHE (Liman *et al.*, *Neuron* 9, 861-71 (1992)) and cloned in *E. coli.* After checking of the sequences, the CHOP-1 DNAs were transcribed *in vitro* (Ambion) and 20 to 30 ng cRNA injected into oocytes from *Xenopus laevis*, as already described for bacteriorhodopsin (Nagel *et al.*, FEBS Letters, 377, 263-266 (1995). The oocytes which expressed the CHOP-1 protein were incubated for two to five days with an *all-trans* retinal (1 µM) in Ringer's solution.

EXAMPLE II

Characterisation of the CHOP-1 Photoreceptor

For the investigation of the supposed ion transport function, the CHOP-1-expressing oocytes were subjected to various experiments using a two-electrode voltage clamp technique which had already been used for bacteriorhodopsin (Nagel et al. FEBS Letters, 377, 263-266 (1995); Nagel *et al.*, *Biophysical Journal* 74, 403-412 (1998)). Green light, but not red light, induced inward-directed currents in oocytes which expressed one of the CHOP-1 RNAs (Fig. 2). The occurrence of photocurrents even with the shorter CHOP-1 RNAs demonstrated that a large part of the carboxy terminal region of CHOP-1 is not necessary for this function. At pH 6 and a transmembrane potential between -100 and + 40 mV the photocurrents were always inward-directed (Fig. 3b). The replacement of chloride by aspartate in the solution had no detectable effect on the amplitude of the photocurrent (Fig. 3a) or its current-voltage ratio (Fig. 3b), a result which ruled out Cl^- as the ion transported. The replacement of sodium by N-methyl-D-glucamine (or of NaCl by sorbitol, data not shown) resulted in a similar inward-directed current (Fig. 3a) with no change in the current-voltage ratio (Fig. 3b), which indicates that Na^+ is not transported by CHOP-

1. Likewise, the replacement of Ca^{2+} by Mg^{2+} gave no change in the photocurrents, a result which showed that Ca^{2+} was also not the ion transported (Fig. 3a,b).

On the other hand, an increase in the proton concentration in the bath solution, $[H^+]_o$, to pH values of 5 and 4 at potentials between -100 and +40 mV resulted in marked increases in the inward-directed photocurrents (Fig. 3a,b).

The results obtained so far thus point to H^+ ions as the charge carriers in the light-induced currents. On account of the sequence homologies between the CHOP-1 protein and the proton pump bacteriorhodopsin mentioned at the outset, it was at first obvious to suppose that the CHOP-1 protein was also a component of an active ion transport system, namely a proton pump such as bacteriorhodopsin.

However, while bacteriorhodopsin always transports protons outwards at all membrane potentials tested from - 60 mV to + 40 mV (see for example Nagel *et al.*, *Biophysical Journal* 74, 403-412 (1998)) even against an existing pH gradient, the transport direction of the CHOP-1 system was dependent on the pH gradient present across the membrane and the membrane potential (Fig. 4a,b,c). The measured reversal potentials at different initial pH gradients (Fig. 4b,c) clearly confirm that the light-induced currents are of a purely passive nature. From the high photocurrents observed, it can be concluded that the proton transport does not proceed only via facilitated diffusion of the protons through the membrane, but rather that the CHOP protein is a proton channel.

The dependence of the light-induced inward-directed photocurrent at pH 5.5 and -40 mV on the wavelength of the light is shown in Fig. 4d. The maximum in the vicinity of 500 nm corresponds to the action spectra for photoreceptor currents, for phototaxia and photoshock reactions of intact *C. reinhardtii* cells.

EXAMPLE III

Amplification of CHOP-2 DNAs and Expression of
Functional CHOP-2 Photoreceptors in *Xenopus laevis*

A full-length CHOP-2 DNA, coding for the amino acids 1-737, and a C-terminally truncated CHOP-2 DNA, coding for the amino acids 1-315 of CHOP-2, were amplified from a full-length cDNA matrix by PCR using a polymerase with correction function (pfu, Promega) and primers which contained BamHI and HindIII restriction sites. The products were inserted into the vector pGEMHE (Liman *et al.*, *Neuron* 9, 861-71 (1992)) and cloned in *E. coli*. After checking of the sequences, the CHOP-2 DNAs were transcribed *in vitro* (Ambion) and 20 to 30 ng cRNA injected into oocytes of *Xenopus laevis*, as already described for bacteriorhodopsin (Nagel *et al.*, FEBS Letters, 377, 263-266 (1995). The oocytes which expressed the CHOP-2 protein were incubated for two to five days with an *all-trans*-Retinal (1 µM) in Ringer's solution.

EXAMPLE IV

Characterisation of the CHOP-2 Photoreceptors

For the investigation of the ion transport function, the CHOP-2-expressing oocytes, which had been incubated with retinal in Ringer's solution for the formation of the ChR2 (Channelrhodopsin2) ion channel were subjected to various experiments (Figs. 5-7) using similar two-electrode voltage clamp techniques, such as had already been used for CHOP-1 and ChR1.

Fig. 5 shows that the light-induced photocurrent on irradiation with steady light (wavelength 450 $\pm$ 25 nm; corresponds roughly to the maximum of the action spectrum) declines to an equilibrium level. This means that the ChR2 ion channel is desensitised by steady light of this wavelength, possibly by inactivation of a part of the ChR2 molecules.

Fig. 6 demonstrates that the ion channel formed with CHOP-2, in contrast to the ion channel formed with CHOP-1, is a non-selective cation channel, which as well as protons also allows various mono and divalent cations to pass. Here the conductivity for the monovalent cations studied decreases in the order $Li^+ > Na^+ > K^+ > Cs^+ >>$

TMA^+ (tetramethylammonium) = TEA^+ (tetraethylammonium), i.e. with increasing ionic radius. With divalent cations, the conductivity also decreases with increasing ionic radius. Here, however, Mg^{2+} is the exception. ChR2 is not permeable to Mg^{2+}, probably on account of the high hydration energy of this ion (Diebler et al., Pure Appl. Chem. 20, p. 93, 1969). At the same time, the respective photocurrents for the ion channel formed with full-length CHOP-2 do not differ from the corresponding photocurrents for the ion channel which contains the CHOP-2 fragment with the amino terminal amino acids 1-315, which allows the conclusion that the cation conductivity is determined by the amino terminal part of the CHOP-2 protein, which contains the seven presumed transmembrane domains.